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3. Full name, address and postcode of the or of each applicant (underline all surnames)

Hannah Research Institute
Ayr
KA6 5HL

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

865333001

4. Title of the invention

"Control of Lactation".

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Murgitroyd & Company
Sootland House
165-169 Scotland Street
Glasgow
G5 8PL

Patents ADP number (if you know it)

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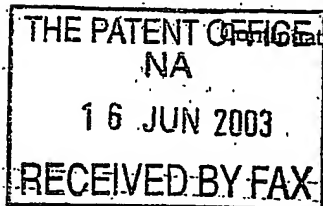
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Continuation sheets of this form

Description

21

Claim(s)

Abstract

Drawing(s)

10

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Statement of inventorship and right to grant of a patent (Patents Form 7/77)

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Gordon Stark

0141 307 8400

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1 "CONTROL OF LACTATION"

2

3 The present invention relates to the identification
4 of three peptides which have a regulatory role in
5 the control of milk secretion. The present
6 invention further provides for the use of the
7 identified peptides and antibodies thereto for the
8 control of milk secretion in lactating animals,
9 including humans.

10

11 Constituents of milk are known to control the rate
12 of milk secretion according to the frequency and
13 completeness with which those constituents are
14 removed through the demand of the offspring or the
15 farmer's husbandry. This biochemical feedback
16 within the breast or udder acts to modulate the
17 lactation-promoting effects of galactopoietic
18 hormones, and its regulatory characteristics, but
19 not all the active factors in milk, have been
20 described by studies on lactating ruminants at the
21 Hannah Research Institute, Ayr, Scotland.
22

1 It has been previously shown that one factor active
2 in goat's and cow's milk is a 7.0-7.6 kDa protein
3 present in a whey protein extract of milk from these
4 animals. This factor was shown to decrease lactose
5 and casein synthesis in cultured explanted pieces of
6 rabbit mammary tissue, and to decrease temporarily
7 the rate of milk secretion when injected into a
8 single mammary gland of the same species via the
9 teat canal.

10
11 These studies did not demonstrate a relationship
12 between the concentration of the 7.0-7.6 kDa protein
13 in cow's milk and the animal's rate of milk
14 secretion, and therefore no pivotal role for this
15 protein in the feedback control of milk secretion by
16 milk removal was demonstrated. It has remained a
17 challenge to determine whether there are other
18 inhibitory factors which are present in cow's milk,
19 and which act to match supply of milk with the
20 demand through a process of feedback inhibition.

21
22 According to a first aspect of the invention there
23 is provided a peptide including the amino acid
24 sequence RPKHPIKHQG (SEQ ID NO: 1), AVAVSQEAN (SEQ
25 ID NO:2) or SEGVALDPAR (SEQ ID NO:3) or an analogue
26 thereof.

27
28 Preferably the peptide is combined with at least one
29 other of the two peptides including the amino acid
30 sequence shown in SEQ ID NO:1, SEQ ID No: 2 or SEQ
31 ID NO:3, this combination reducing milk secretion in
32 animals, including humans.

1. Preferably the amino acid sequence shown in SEQ ID
2. NO:1, SEQ ID No: 2 and SEQ ID NO:3 is the N-terminal
3. sequence of the peptide.

4.
5. Preferably the peptide can be co-purified with each
6. of the other peptides including the amino acid
7. sequence shown in SEQ ID NO:1, SEQ ID No: 2 or SEQ
8. ID NO:3 from a 6-30 kDa fraction of whey protein of
9. cow's milk.

10.
11. In particular, the peptides can be purified from
12. cow's milk by a series of chromatographic separation
13. techniques.

14.
15. Specifically, when a 6-30 kDa fraction of the whey
16. proteins of cow's milk is resolved by gel filtration
17. on a cross-linked copolymer of allyl dextran and N,N
18. methylenebisacrylamide having an average particle
19. size of 47 μ m, such as Sephacryl S-100 (Pharmacia).
20. The fourth-eluting component resolved by this
21. method, "peak S4", comprises the inhibitory
22. peptides.

23.
24. More specifically, the peptides are co-purified when
25. a nominally 6-30 kDa fraction of the whey proteins
26. of cow's milk is resolved by gel filtration on a
27. cross-linked copolymer of allyl dextran and N,N
28. methylenebisacrylamide having an average particle
29. size of 47 μ m, such as Sephacryl S-100 (Pharmacia).
30. The fourth-eluting component resolved by this
31. method, "peak S4", comprises the peptides. When
32. peak S4 is resolved further by peptide gel-

1 filtration chromatography on a gel of dextran
2 covalently bonded to highly cross-linked agarose
3 beads with a mean diameter of 13-15 μ m, such as
4 Superdex Peptide HR (Pharmacia), the leading edge of
5 the major eluted component eluting at 8-11.5 ml,
6 designated P8-11A, contains the inhibitory peptides.
7 Further, when fraction P8-11A is resolved by
8 reversed phase chromatography on a reversed phase
9 column (Genesis 25 cm, C18 4micron; Jones
10 Chromatography), the fractions eluted after 34-36
11 min at a concentration of 36-39% acetonitrile, in a
12 linear gradient of same in 0.1% trifluoroacetic
13 acid, contains the peptides.

14
15 There is further provided a peptide including the
16 amino acid sequence shown in SEQ ID NO:1, SEQ ID No:
17 2 or SEQ ID NO:3, which in combination with one or
18 more of the other peptides including the amino acid
19 sequence shown SEQ ID NO:1, SEQ ID No: 2 or SEQ ID
20 NO:3 provides a reduction in milk secretion.

21
22 In one preferred embodiment, the peptide includes
23 the amino acid sequence shown in SEQ ID NO:1 or an
24 analogue thereof.

25
26 In another preferred embodiment, the peptide
27 includes the amino acid sequence shown in SEQ ID
28 NO:2 or an analogue thereof.

29
30 In a yet further preferred embodiment, the peptide
31 includes the amino acid sequence shown in SEQ ID
32 NO:3 or an analogue thereof.

5

1 A further aspect of the invention provides a peptide
2 mixture comprising two or more different peptides,
3 the peptides including the amino acid sequence shown
4 SEQ ID NO:1, SEQ ID No: 2 or SEQ ID NO:3, or
5 analogues thereof.

6
7 Preferably the peptide has a molecular mass
8 determined by mass spectrometric analysis of between
9 1000 to 3000 Da.

10
11 In a particular embodiment of the invention, the
12 peptide is glycosylated.

13
14 Alternatively the peptide is unglycosylated.

15
16 Further, the peptides of the present invention can
17 be in either phosphorylated or unphosphorylated
18 form.

19
20 The present invention further includes truncated
21 versions of the peptides which have been isolated
22 from milk.

23
24 Analogues of and for use in the invention as defined
25 herein means a peptide modified by varying the amino
26 acid sequence e.g. by manipulation of the nucleic
27 acid encoding the protein or by altering the protein
28 itself. Such derivatives of the amino acid sequence
29 may involve insertion, addition, deletion and/or
30 substitution of one or more amino acids, while
31 providing a peptide capable of influencing milk

6

1 secretion either on its own, or in combination with
2 other peptides.

3

4 Preferably such analogues involve the insertion,
5 addition, deletion and/or substitution of 10 or
6 fewer amino acids, more preferably of 5 or fewer,
7 and most preferably of only 1 or 2 amino acids.

8

9 Analogues also include derivatives of the defined
10 peptides, including the peptide being linked to a
11 coupling partner, e. g. an effector molecule, a
12 label, a drug, a toxin and/or a carrier or transport
13 molecule. Techniques for coupling the peptides of
14 the invention to both peptidyl and non-peptidyl
15 coupling partners are well known in the art.

16

17 A second aspect of the present invention provides a
18 method of influencing milk secretion in animals, the
19 method including the steps of administering at least
20 one peptide according to the first aspect of the
21 invention.

22

23 Preferably the term animal is taken to include
24 humans.

25

26 In one preferred embodiment, the animal is a cow,
27 goat or sheep.

28

29 A yet further aspect of the present invention
30 provides for antibodies directed to the peptides
31 including the amino acid sequence shown in SEQ ID
32 NO:1, SEQ ID No: 2 or SEQ ID NO:3.

1 Preferably said antibodies promote or improve
2 lactation in humans and other mammals.
3

4 Preferably said antibodies promote or improve
5 lactation in sheep, cows and goats.
6

7 An "antibody" is an immunoglobulin, whether natural
8 or partly or wholly synthetically produced. The
9 term also covers any polypeptide, protein or peptide
10 having a binding domain which is, or is homologous
11 to, an antibody binding domain. These can be
12 derived from natural sources, or they may be partly
13 or wholly synthetically produced. Examples of
14 antibodies are the immunoglobulin isotypes and their
15 isotypic subclasses and fragments which comprise an
16 antigen binding domain such as Fab, scFv, Fv, dAb,
17 Fd; and diabodies.
18

19 The binding member of the invention may be an
20 antibody such as a monoclonal or polyclonal
21 antibody, or a fragment thereof. The constant region
22 of the antibody may be of any class including, but
23 not limited to, human classes IgG, IgA, IgM, IgD and
24 IgE. The antibody may belong to any sub class e.g.
25 IgG1, IgG2, IgG3 and IgG4.
26

27 As antibodies can be modified in a number of ways,
28 the term "antibody" should be construed as covering
29 any binding member or substance having a binding
30 domain with the required specificity. Thus, this
31 term covers antibody fragments, derivatives,
32 functional equivalents and homologues of antibodies,

1 including any polypeptide comprising an
2 immunoglobulin binding domain, whether natural or
3 wholly or partially synthetic. Chimeric molecules
4 comprising an immunoglobulin binding domain, or
5 equivalent, fused to another polypeptide are
6 therefore included. Cloning and expression of
7 chimeric antibodies are described in EP-A-0120694
8 and EP-A-0125023.

9
10 It has been shown that fragments of a whole antibody
11 can perform the function of antigen binding.

12
13 Examples of such binding fragments are (i) the Fab
14 fragment consisting of VL, VH, CL and CH1 domains;
15 (ii) the Fd fragment consisting of the VH and CH1
16 domains; (iii) the Fv fragment consisting of the VL
17 and VH domains of a single antibody; (iv) the dAb
18 fragment (Ward, E.S. et al., Nature 341:544-546
19 (1989)) which consists of a VH domain; (v) isolated
20 CDR regions; (vi) F(ab')₂ fragments, a bivalent
21 fragment comprising two linked Fab fragments (vii)
22 single chain Fv molecules (scFv), wherein a VH
23 domain and a VL domain are linked by a peptide
24 linker which allows the two domains to associate to
25 form an antigen binding site (Bird et al., Science
26 242:423-426 (1988); Huston et al., PNAS USA 85:5879-
27 5883 (1988)); (viii) bispecific single chain Fv
28 dimers (PCT/US92/09965) and (ix) "diabodies",
29 multivalent or multispecific fragments constructed
30 by gene fusion (WO94/13804; P. Hollinger et al.,
31 Proc. Natl. Acad. Sci. USA 90:6444-6448 (1993)).
32

1 The term "antibody" includes antibodies which have
2 been "humanised". Methods for making humanised
3 antibodies are known in the art. Methods are
4 described, for example, in Winter, U.S. Patent No.
5 5,225,539. A humanised antibody may be a modified
6 antibody having the hypervariable region of a
7 monoclonal antibody such as 791T/36 and the constant
8 region of a human antibody. Thus the binding member
9 may comprise a human constant region.

10
11 A yet further aspect of the present invention
12 provides a composition for influencing lactation in
13 animals, the composition including a peptide
14 including the amino acid sequence RPKHPIKHQG (SEQ ID
15 NO: 1), AVAVSQEAN (SEQ ID NO:2) or SEGVALDPAR (SEQ
16 ID NO:3) or an analogue thereof.

17
18 Preferably the animal is a non-human animal.

19
20 More preferably the animal is a cow, goat or sheep.

21
22 Preferably the composition inhibits lactation in the
23 target cells within hours of administration, with
24 the response dependent on the frequency of milk
25 removal from the mammary gland.

26
27 Preferably the composition is administered by intra-
28 ductal injection into the mammary gland at a dose
29 level yielding a final concentration of peptides in
30 milk in the range 0.1 to 10 micromolar. The
31 administration of this dose may be repeated as

1 required, and possibly increased when given over
2 long periods.
3

4 Preferred features of each aspect of the invention
5 are as for each of the other aspects *mutatis*
6 *mutandis*.
7

8 In summary, the present invention provides the
9 surprising and unexpected finding that at least one
10 peptide which can act in combination with other
11 defined peptides, inhibits the secretion of milk
12 constituents in primary cell cultures that reproduce
13 the activities of lactating mammary tissue.
14

15 Description of the drawings
16

17 The present invention will now be described, by way
18 of example only, with reference to the accompanying
19 drawings, wherein;
20

21 Figure 1 shows the resolution of the 6-30 kDa
22 whey fraction by Sephacryl gel filtration
23 chromatography,
24

25 Figure 2 shows the further resolution of gel-
26 filtration peak S4 by Superdex peptide
27 chromatography,
28

29 Figure 3 shows an example of the further
30 resolution of the Superdex fraction P8-11A by
31 reversed phase HPLC,
32

1 Figure 4 shows the inhibition of protein
2 secretion in acini cultures by fractions obtained
3 through resolution of a 6-30 kDa fraction of cow's
4 whey proteins by Sephacryl gel filtration
5 chromatography;

6
7 Figure 5 shows the inhibition of protein
8 secretion in cell culture by fractions of prepared
9 by Superdex high-resolution peptide chromatography,
10

11 Figure 6 shows inhibition of protein secretion
12 in mammary acini cultures by components of peptide
13 fraction 8-11A resolved by reversed phase HPLC,
14

15 Figure 7 shows the effect of peptide A on
16 protein secretion in mammary cell cultures,
17

18 Figure 8 shows the effect of peptide B on
19 protein secretion in mammary cell cultures,
20

21 Figure 9 shows the effect of peptide C on
22 protein secretion in mammary cell cultures, and
23

24 Figure 10 shows the effect of a combination of
25 peptides A, B and C on protein secretion in mammary
26 cell cultures.

27
28 Detailed Description

29
30 The peptides of the invention exist in cow's milk,
31 possibly in glycosylated or phosphorylated form.

1 The peptides may act together to inhibit the rate of
2 milk secretion in the mammary gland.
3

4 The peptides of the invention can be obtained from
5 cow's milk by a method described herein or by some
6 variant thereon. It has been demonstrated that the
7 three peptides of the invention isolated from cow's
8 milk are able to inhibit the secretion of milk
9 proteins in mammary acini cultures. When the three
10 peptides are present together in a milk fraction
11 added to the culture medium for a two hour period,
12 they are able to inhibit protein secretion in a
13 concentration-dependent manner.
14

15 Synthetic peptides based on the N-terminal sequence
16 of the natural peptides can be synthesised by
17 standard Fmoc amino acid chemistry. Synthetic
18 peptides produced according to the N-terminal
19 sequence of the peptides of the invention, and
20 representing truncated 9- or 10-amino acid forms of
21 the natural peptides similarly inhibit the secretion
22 of protein in primary cultures of mammary cells
23 prepared as acini by collagenase digestion of
24 lactating tissue. Inhibition is exerted acutely,
25 within two hours, and is elicited in a
26 concentration-dependent manner by synthetic peptides
27 in combination. It is expected that the inhibitory
28 activity of synthetic peptides will depend on the
29 proportion of the full-length sequence synthesised
30 and that the inhibitory potency of these and the
31 natural peptides will depend on the degree of

1 peptide modification by glycosylation or
2 phosphorylation.
3

4 The invention is applicable to any animal responsive
5 to the inhibitory peptides defined herein. In
6 addition, the demand-led relationship between milk
7 supply and milk removal in most if not all mammals
8 predicts that the same effects will be demonstrable
9 in relation to the peptides of the invention
10 obtained from milk of other species, in relation to
11 that species. In man, administration by a suitable
12 route of the peptides, or antibodies thereto, may be
13 applied to improve or suppress lactation. In dairy
14 cows, there maybe a need to reduce milk yield in
15 order to maintain production within quota limits, in
16 which case the inhibitory peptides themselves are
17 administered. For intra-ductal injection of
18 peptides into the mammary gland, a dose yielding a
19 final concentration of peptides in milk in the range
20 0.01-1.0 micromolar is likely to be effective, and
21 should be repeated as required, and possibly
22 increased when given over long periods.
23

24 Conversely, passive immunisation methods using
25 antibodies against the peptides may be used to
26 generate a reduction in the effect of the natural
27 inhibitory peptides when this is desired in order to
28 increase milk supply in lactating animals.
29

30 Antibodies against the natural peptides of the
31 invention or against their synthetic analogues can
32 be raised by conventional methods e.g. as polyclonal

1 antisera, mouse monoclonal antibodies, cow-mouse
2 hybrid monoclonal antibodies or as engineered
3 antibodies, by any of the currently available
4 methods: Conventional carriers and adjuvants known
5 in vaccination can be used. Antibodies against

6 synthetic truncated peptides based on the sequence
7 of the peptides of the invention may be used to
8 isolate the natural peptides from cow's milk
9 extracts, or to control milk supply as described
10 above.

11

12 EXAMPLE

13 Preparation of cow milk fractions

14

15 Milk was collected at the morning milking from
16 Friesian cows, and was defatted by centrifugation
17 (2500g, 15°C, 20 min) and filtered through glass
18 wool. Casein in defatted milk was precipitated by
19 dropwise addition of concentrated HCl until the pH
20 reached 4.6. After stirring for 10 min, casein was
21 sedimented by centrifugation (2500g, 15 °C, 20 min),
22 and the clear whey supernatant was filtered through
23 glass fibre membranes of decreasing pore size, the
24 final membrane made of polyethersulphone having a
25 cut-off of 0.45 microns. The whey fraction was
26 subjected to ultrafiltration using a filter with a
27 nominal cut-off value of molecular weight 30,000
28 Daltons (Da). The filtrate was dialysed for 24 h
29 against 10 mM sodium acetate buffer pH 4.6
30 containing 1.5mM ϵ -aminocaproic acid, 100 μ M
31 glutathione, 1mM EDTA and 1mM EGTA using a dialysis
32 membrane with a nominal molecular weight cut-off of

1 6,000 Da, and was then adjusted to pH 7.0 by
2 addition of NaOH. The neutralised filtrate was
3 dialysed against 2mM phosphate buffer pH 7.0
4 containing 1.5mM ϵ -aminocaproic acid, 100 μ M

5 glutathione, 1mM EDTA and 1 mM EGTA for 24 h and
6 then freeze dried.

7 8 Gel Filtration Chromatography

9
10 The 6-30kDa whey fraction was resolved of a Hi-Prep
11 Sephacryl S-100 High-Resolution gel filtration
12 column (Pharmacia) using a Fast Protein Liquid
13 Chromatography (FPLC) system (Pharmacia). The
14 freeze-dried whey fraction was reconstituted in one
15 tenth its volume before freeze-drying, and the
16 solution was clarified by filtration through a 0.22
17 μ m filter. The chromatography buffer was 20 mM
18 phosphate buffer pH 7.0 containing 0.15 M NaCl, and
19 was filtered through a 0.22 μ m filter and degassed
20 before use. Two ml of the 10 x concentrated whey
21 fraction was loaded for each separation. The flow
22 was 1ml/min.

23
24 Fractions containing protein peaks eluted from the
25 column were tested for inhibitory activity in a cell
26 culture bioassay (see below), and fractions spanning
27 one protein peak containing inhibitory activity,
28 designated peak S4, were combined and desalted by
29 passage through a column composed of Poros 50 R2,
30 composed of cross-linked poly(styrene-divinylbenzene
31 (PerSeptive Biosystems). Protein bound to the
32 column was washed with distilled water, and then

1 eluted with 80% (v/v) acetonitrile in distilled
2 water. The inhibitory fraction was then
3 concentrated under a stream of nitrogen and freeze
4 dried. A second peak of inhibitory material,
5 designated peak S6, contained too little protein for
6 further purification to be practicable.

7
8 Fraction S4 was resolved further on a Superdex
9 Peptide HR 10/30 column (Pharmacia) using an FPLC
10 system. Dried fraction was reconstituted in 0.2-0.4
11 ml of solution of 0.2 mM AEBSF, 10 mM EDTA and 10 mM
12 EGTA, and chromatography was performed in 20 mM
13 phosphate buffer pH 7.0 containing 0.25 M NaCl at a
14 flow rate of 1 ml/min. Fractions containing protein
15 peaks eluting from the column were collected. These
16 were either desalted on a Poros column and freeze
17 dried as described above for assay of inhibitory
18 activity, or were freeze dried immediately for
19 further fractionation. Inhibitory material was
20 detected in the leading edge of the major peak,
21 designated fraction P8-11A.

22
23 Fraction P8-11A was resolved further using a
24 reversed phase HPLC column Genesis 25 cm C18, 4 μ ;
25 Jones chromatography) on a Spectra Physics HPLC
26 system. Sample containing the equivalent of one
27 Superdex fraction P8-11A was dissolved in water and
28 loaded in a volume of 0.2 ml. The column was eluted
29 with a gradient of 0-60% acetonitrile in 0.1% (w/v)
30 trifluoroacetic acid (TFA), and 1 ml fractions of
31 the eluate were collected. HPLC fractions were

17

1 freeze dried, and tested for inhibitory activity as
2 described above.

3

4 Mammary cell culture bioassay of milk fractions

5

6 Mammary cells were prepared from tissue of lactating
7 mice by collagenase digestion according to the
8 method of K Hendry, K Simpson, K Nicholas & C Wilde.
9 Journal of Molecular Endocrinology 21: 169-177
10 (1998). The resultant suspension of mammary acini
11 consisted predominantly of groups of 50-200 cells,
12 and was cultured in medium (Medium 199/Ham's F12:
13 50:50 v/v) containing insulin (5 µg/ml),
14 hydrocortisone (0.1 µg/ml) and prolactin (1 µg/ml).
15 Culture density was 1.5×10^6 cells/ml, and cells
16 were maintained at 37°C in an atmosphere of 5% CO₂ in
17 air. Protein synthesis and secretion were measured
18 by continuous labelling with L-[4,5-³H]leucine (40-
19 70 mCi/mmol; 10-20 µCi/ml) for 2 h in the presence
20 or absence of milk fractions at concentrations of
21 0.2 - 4.0 µg/ml) or synthetic peptides (0.01 - 10
22 µM). Milk extracts or synthetic peptide were
23 dissolved and diluted in 10 mM Hepes buffer pH 7.4,
24 and control cultures containing only diluent were
25 included in each experiment. The culture was
26 terminated by centrifugation of the cell suspension
27 (2000g, 2 min), and the cell pellet and supernatant
28 were frozen and stored separately for assay of DNA
29 and protein secretion respectively. Radiolabel
30 incorporation was measured by precipitation with
31 trichloroacetic acid (final concentration 10% w/v).
32 Cell lysates were prepared by sonication using a

1 Kontes KT50 cell disrupter (setting 20, 15 s) in 0.1
2 M NaH_2PO_4 pH 7.4 containing 2 M NaCl, and assayed for
3 DNA content by the method of C Labarca and K Paigen,
4 *Analytical Biochemistry* 102: 344-352 (1980).

5 Secretory activity was expressed per unit of
6 cellular DNA.

7
8 The amount of radiolabelled protein secreted by
9 acini in the presence of milk extracts or synthetic
10 peptides was expressed as a percentage of that
11 produced by the cells to which no milk fraction or
12 peptide was added. In each experiment, treatments
13 were replicated in a minimum of three culture wells,
14 and results for individual experiments were mean
15 values for those wells. Values shown in Figures 4-6
16 are the mean for three or four experiments, each
17 testing a different preparation of milk fractions.
18 Error bars show the standard error of the mean for
19 these experiments.

20
21 Figure 4 shows the inhibition of protein secretion
22 in acini cultures by fractions obtained through
23 resolution of a 6-30 kDa fraction of cow's whey
24 proteins by Sephacryl gel filtration chromatography.
25 Figure 5 shown the inhibition of protein secretion
26 in cell culture by fractions of prepared by Superdex
27 high-resolution peptide chromatography.
28 Figure 6 shown inhibition of protein secretion in
29 mammary acini cultures by components of peptide
30 fraction 8-11A resolved by reversed phase HPLC.
31

19

1 Preparation of synthetic peptides based on natural
2 peptide sequences

3

4 N-terminal sequencing of the inhibitory HPLC

5 fraction eluting at 36-39% acetonitrile revealed
6 three peptides with the N-terminal sequences:

7

8 Peptide A: RPKHPIKHQG

9 Peptide B: AVASQEAN

10 Peptide C: SEGVALDPAR

11

12 Peptide A was identified as the N-terminal sequence
13 of α_{s1} -casein. Peptides B and C have limited
14 homology to known amino acid sequence data
15 (SwissProt protein sequence database). Mass
16 spectrometric analysis indicated that peptides had
17 masses in the range 1000 - 3000 Daltons.

18

19 Peptide A was produced synthetically by chymosin
20 digestion of α_{s1} -casein purified by a modification of
21 the method of E. Lahov and W Regelson, Food
22 Chemistry and Toxicology 34:1 131-145 1996. The N-
23 terminal fragment of mass 2762 was isolated from the
24 chymosin digest by reversed phase chromatography
25 system. This peptide consists of a 23 amino acid
26 sequence at the N-terminus of the α_{s1} -casein protein.

27

28 N-terminal sequences of Peptides B and C comprising
29 nine and ten amino acids respectively were
30 synthesised by solid phase synthesis using Fmoc
31 amino acids coupled by the PyBOP/HOBt/DIPEA method.
32 The peptides were cleaved from the resin with 80%

1 TFA plus suitable scavengers and purified by reverse.
2 phase HPLC on a Phenomenex Luna 10 μ C18 column of
3 dimensions 25cm x 0.212cm using a linear gradient of
4 water to 100% acetonitrile in 0.1% TFA.

5 -----
6 **Mammary culture bioassay of synthetic peptides**
7

8 Protein secretion was measured in mammary acini
9 cultures in the presence and absence of synthetic
10 peptides. Peptides were added singly or in
11 combination at equimolar concentrations over a range
12 of 0.01 - 10.0 μ M. In each experiment, treatments
13 were replicated in a minimum of three culture wells,
14 and results for individual experiments were mean
15 values for those wells. Values shown in Figures 7-9
16 are the mean for three experiments. Error bars show
17 the standard error of the mean for these
18 experiments.
19

20 Statistical analysis of bioassay data showed that,
21 together, the three peptides exhibited a
22 concentration-dependent inhibition of secretion.
23 Maximal inhibition of secretion was obtained at a
24 concentration of 0.1-1.0 μ M of each peptide. None of
25 the peptides tested individually inhibited secretion
26 to this extent, and at higher concentrations the
27 inhibitory action of peptide A was counteracted by a
28 stimulatory effect of peptide B. The effects of the
29 synthetic peptides indicate that inhibition of
30 secretion by the HPLC fraction containing natural
31 peptides with the same N-terminal sequences is due
32 to the combined actions of the peptides, and is

1 unlikely to be conferred by any one constituent of
2 the active HPLC fraction.

3

4 Figures 7, 8 and 9 show the effect of peptides A, B
5 ~~and C on protein secretion in mammary cell cultures.~~

6 Figure 10 shows the effect of a combination of
7 peptides A, B and C on protein secretion in mammary
8 cell cultures.

9

10 All documents referred to in this specification are
11 herein incorporated by reference. Various
12 modifications and variations to the described
13 embodiments of the inventions will be apparent to
14 those skilled in the art without departing from the
15 scope of the invention. Although the invention has
16 been described in connection with specific preferred
17 embodiments, it should be understood that the
18 invention as claimed should not be unduly limited to
19 such specific embodiments. Indeed, various
20 modifications of the described modes of carrying out
21 the invention which are obvious to those skilled in
22 the art are intended to be covered by the present
23 invention.

1/10

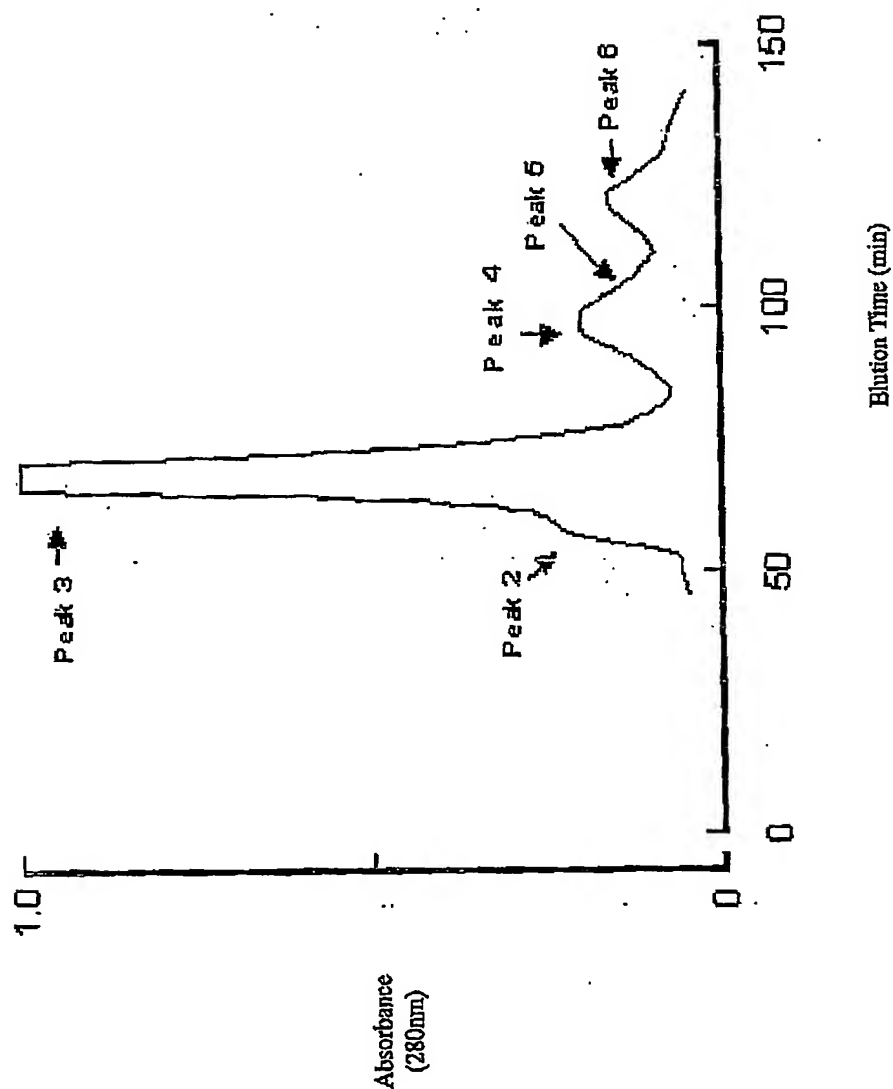


Figure 1.
Gel filtration chromatography of bovine 6-30 kDa whey fraction

2/10

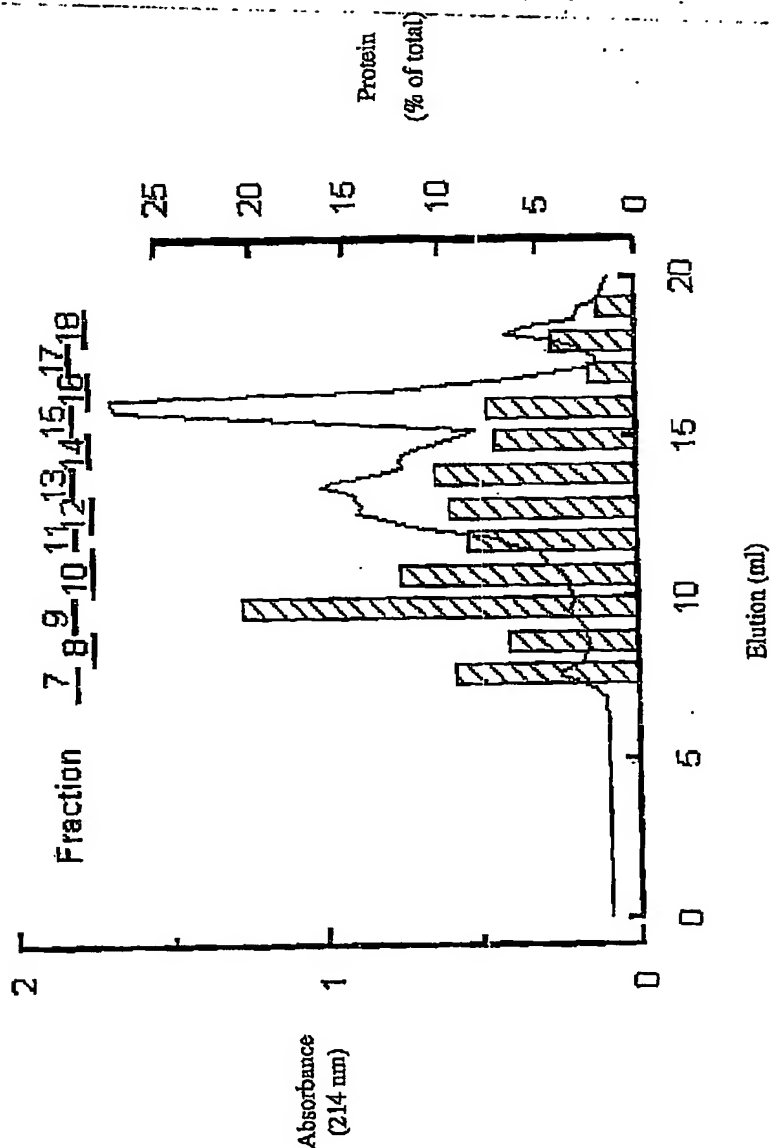


Figure 2. Resolution of inhibitory peak S4 by peptide chromatography

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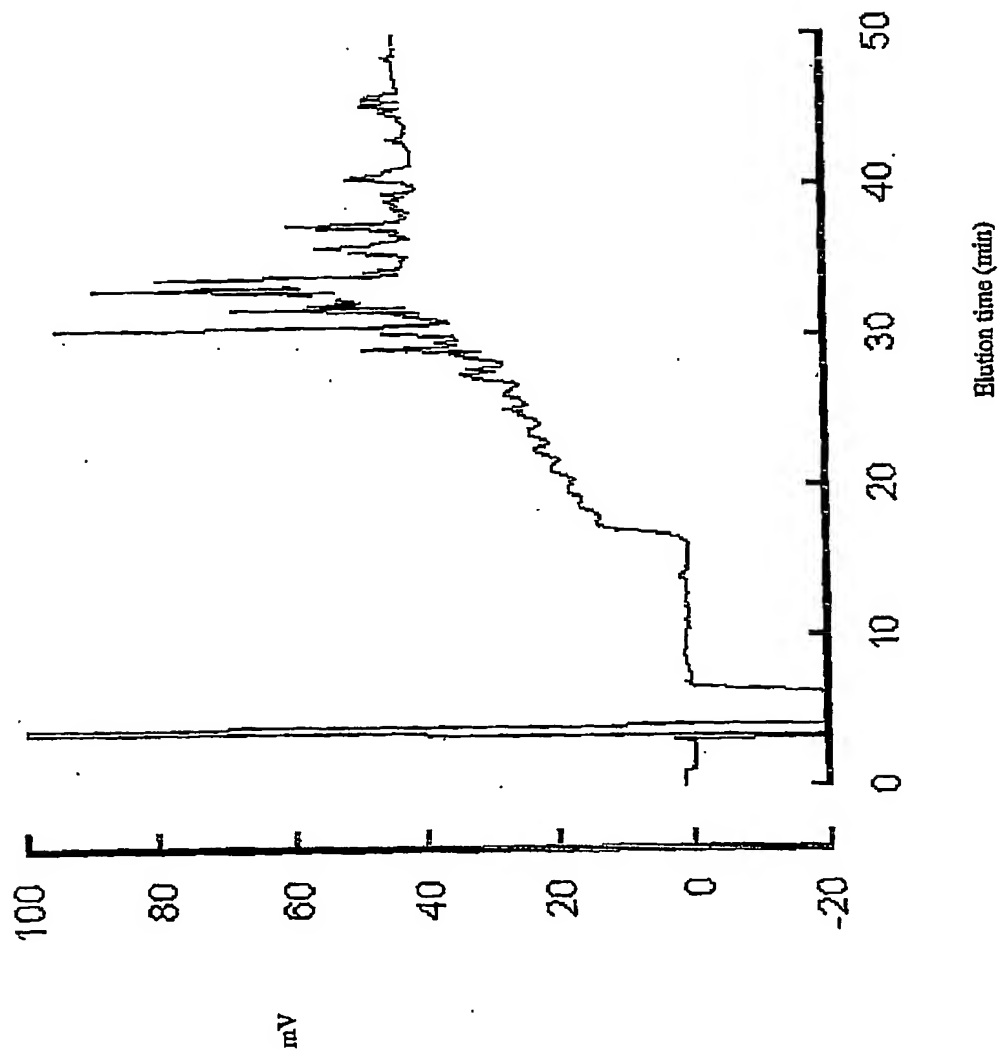


Figure 3. Resolution of peptide fraction P8-11A by reversed phase chromatography

4/10

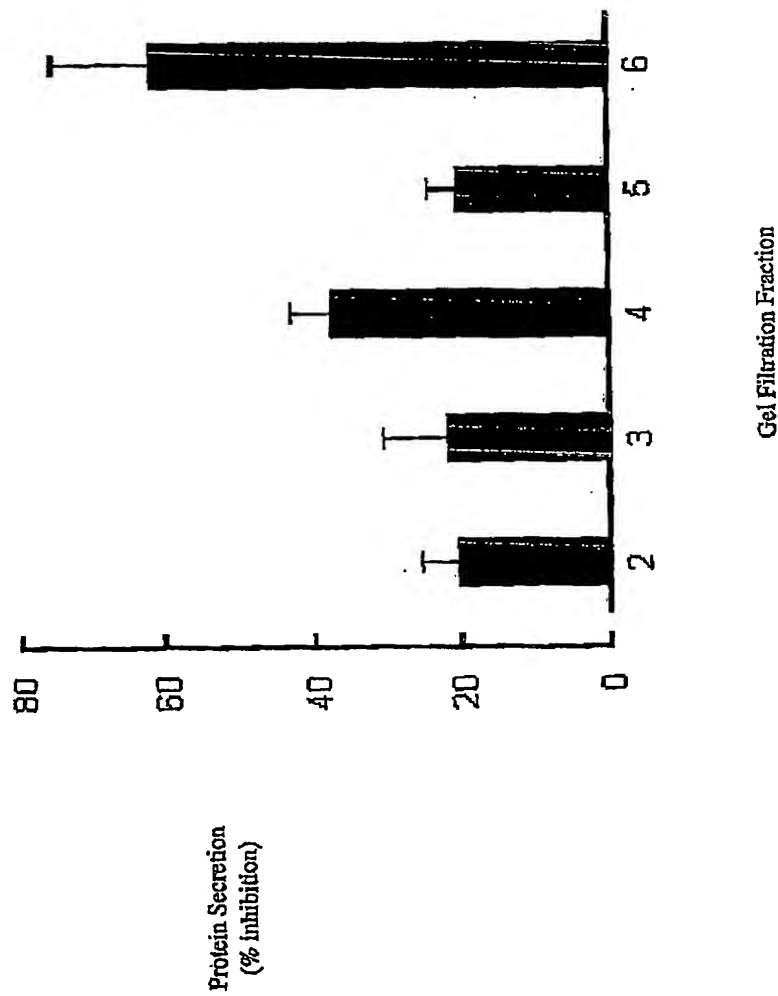


Figure 4. Bioassay of gel-filtered whey fractions

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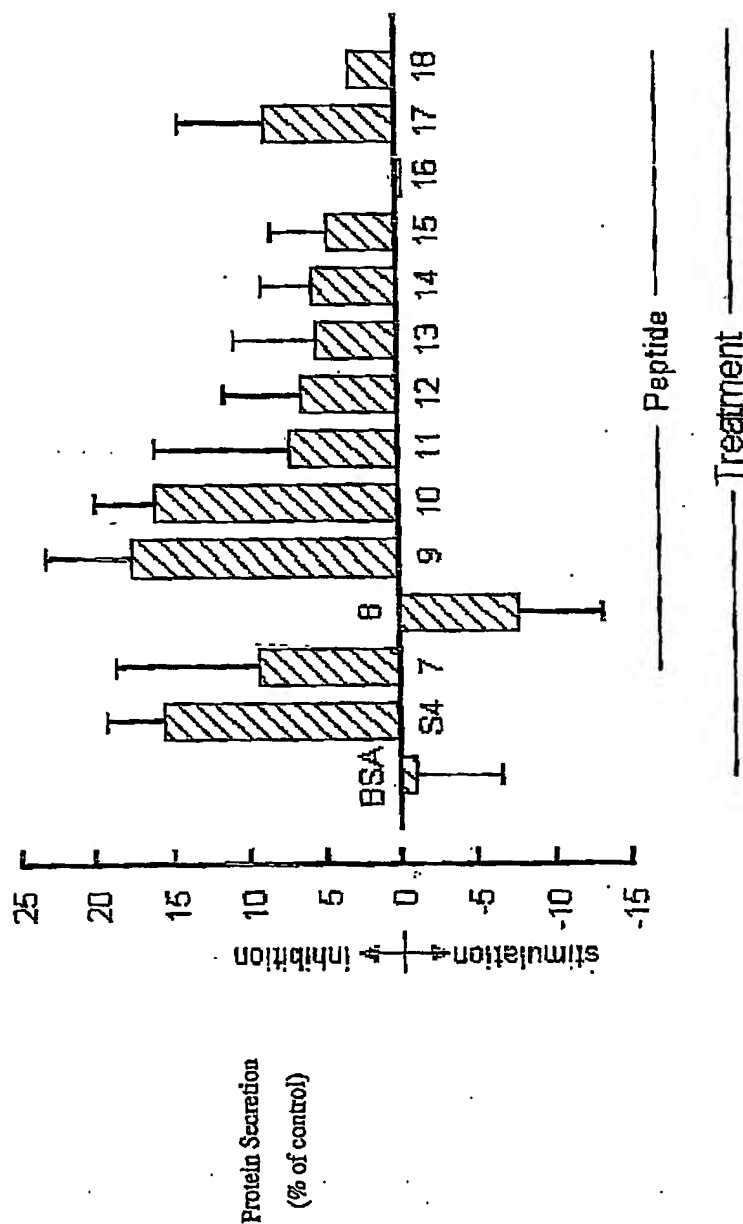


Figure 5. Inhibition of protein secretion by bovine whey peptide fractions

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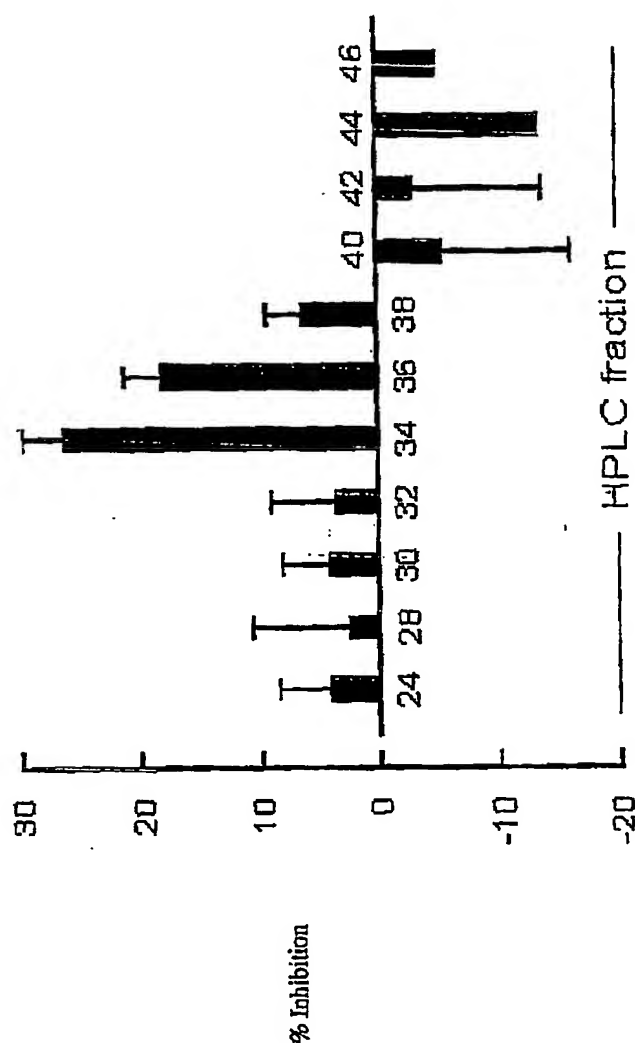


Figure 6. Inhibition of protein secretion by HPLC-resolved fractions

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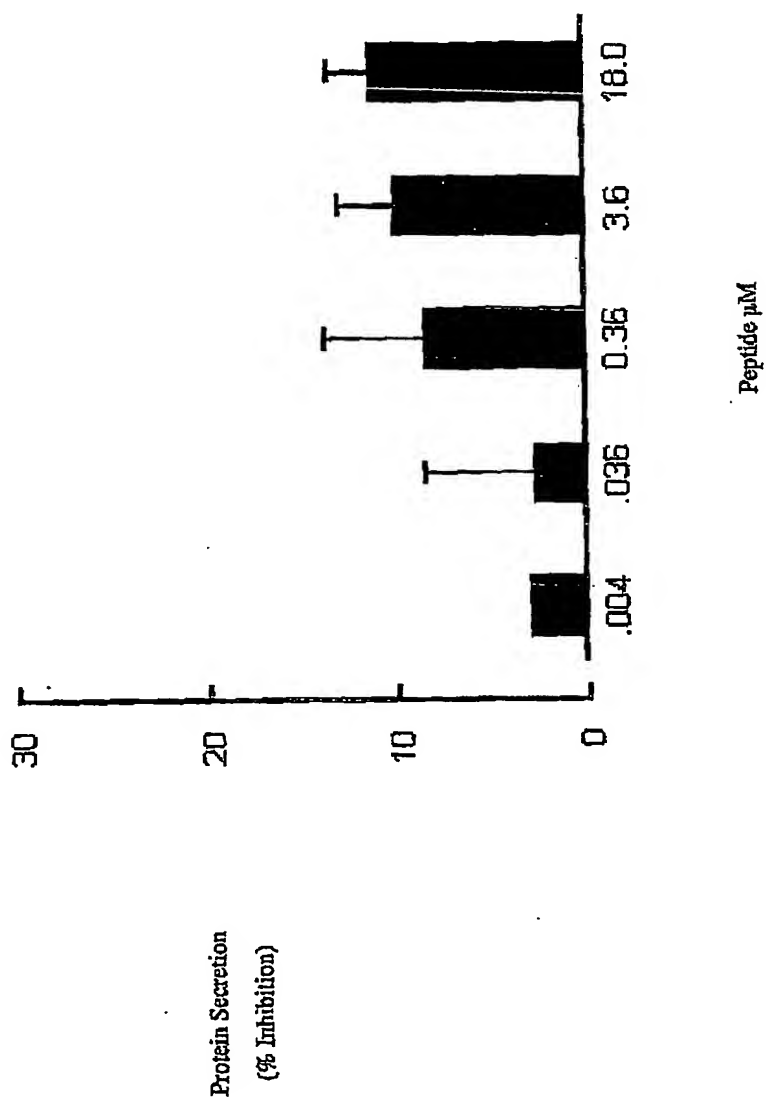


Figure 7. Inhibition of protein secretion in mammary cell cultures by synthetic peptide A

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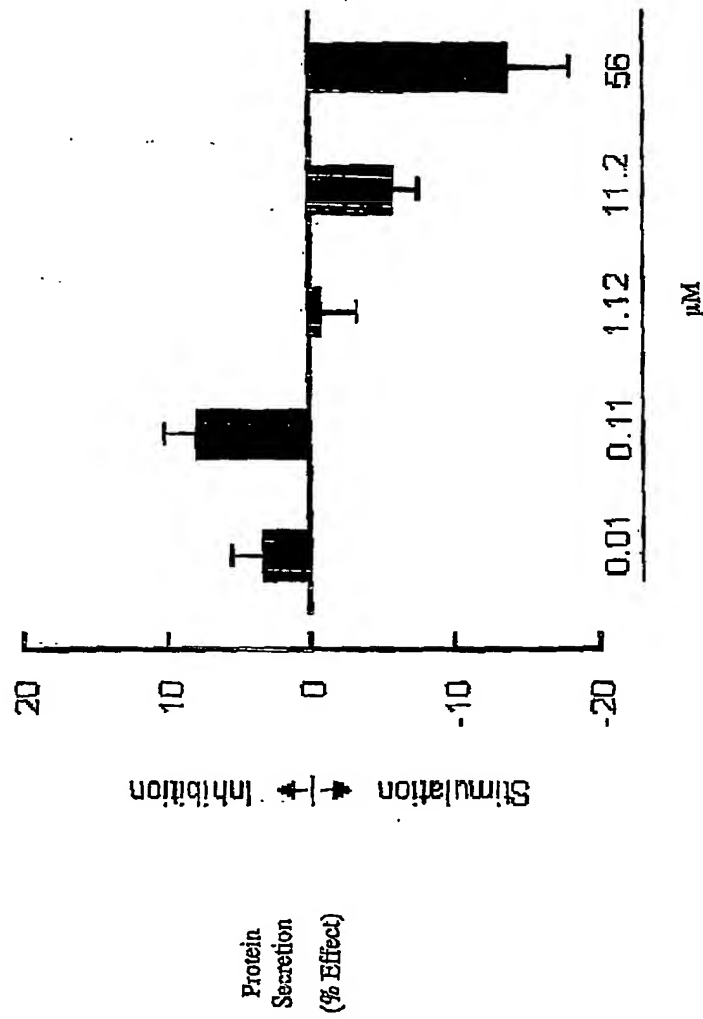


Figure 8. Inhibition of protein secretion in mammary cell cultures by synthetic peptide B

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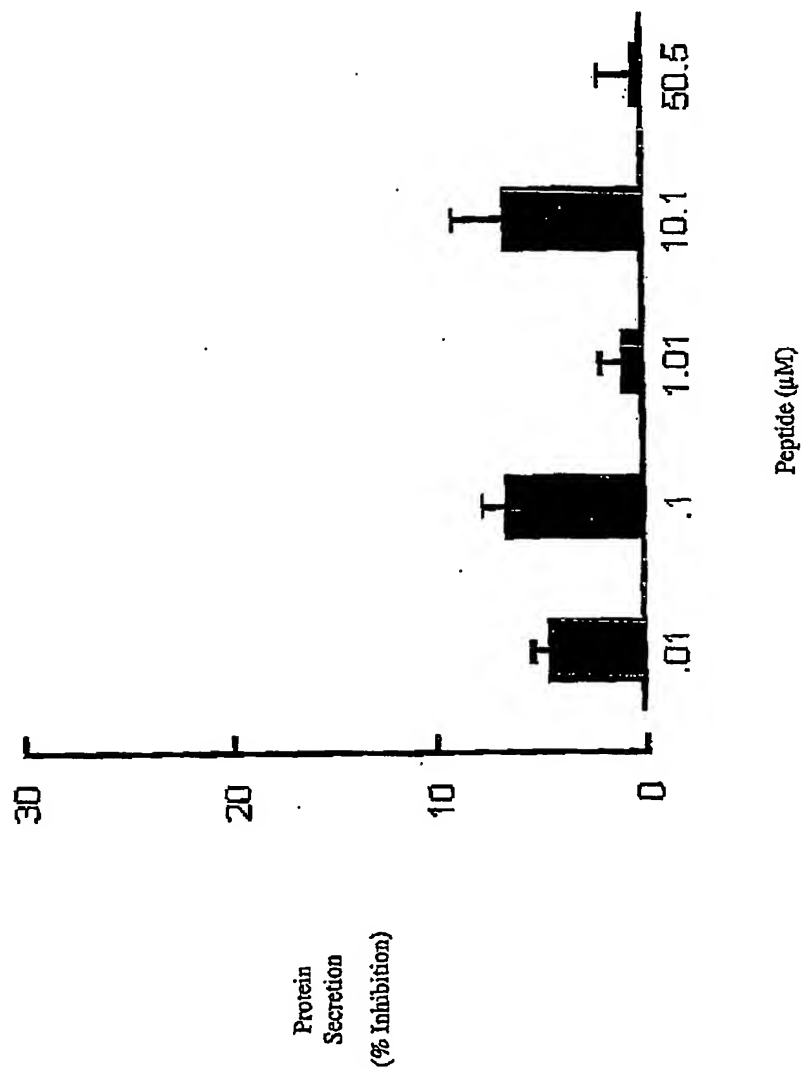


Figure 9. Inhibition of protein secretion in mammary cell cultures by peptide C

10/10

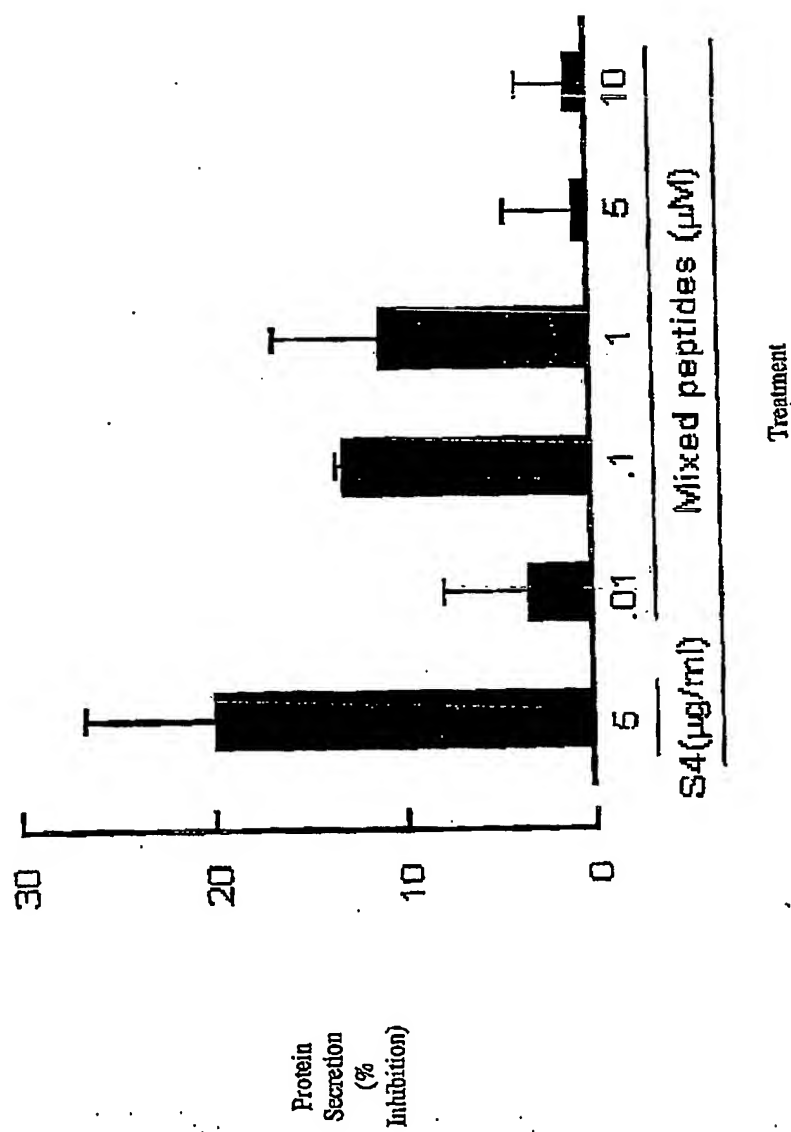


Figure 10. Inhibition of protein secretion in mammary cell cultures by a combination of synthetic peptides A, B and C.

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